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Sclerotial Development of *Grifola umbellata*

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Sclerotial development of *Grifola umbellata* (Pers. : Fr.) Donk was investigated through microscopic examinations. The sclerotium of *G. umbellata* was bumpy and rugged, multi-branched, and dark-brown to black in color. The sclerotial development of *G. umbellata* was categorized into three stages such as sclerotial initial, development and maturation. Sclerotium development was initiated as the white fungal mass. The superficial part of white sclerotium changed into gray, light brown and then black as its development proceeded further. As a distinctive characteristic of this fungus, a large number of crystals were observed in the medulla layer of sclerotium during its maturation. For development of new sclerotium, *G. umbellata* formed a white sclerotial primordium on the matured sclerotium. Development of sclerotium in *G. umbellata* was intimately associated with rhizomorphs of *Armillariella mellea* and the developing sclerotia were often penetrated by rhizomorphs of *A. mellea* into medulla layer.

KEYWORDS: ***Armillariella mellea, Grifola umbellata*, Rhizomorph, Sclerotial development**

Grifola umbellata (Pers. : Fr.) Donk belongs to the family Polyporaceae of Basidiomycota, and has been considered as a species of wood rotting fungi. This fungus forms an underground tuber-like structure such as sclerotium in its life cycle. General features of its sclerotium have been known as a multi-branched and irregular tuber in form, black in color and woody in texture (Mao and Jiang, 1993; Imazeki and Hongo, 1989). Particularly, the sclerotium of *G. umbellata* has been used for medicinal purposes in oriental countries, especially in Korea, China and Japan. The sclerotium of this fungus possesses potent effects for promoting a diuretic process (Lu *et al.*, 1985) and hair growth (Inaoka *et al.*, 1994), and suppressing cytotoxic activity of leukemia (Ohsawa *et al.*, 1992) and other cancers (You *et al.*, 1994). In these regards, *G. umbellata* has a potential value in global market as the important medicinal fungus. Since sclerotia of *G. umbellata* have not been produced in Korea, the domestic needs for the sclerotia have been depending on the imports from China, and have been increased year after year. Also, there have been few studies on the mass production of sclerotia of *G. umbellata* as well as its mycological aspects in Korea. In these regards, we tried to elucidate the details of sclerotial development in *G. umbellata.* This study will not only reveal the insight of the sclerotial development of *G. umbellata* but also contribute to establishing the basic knowledge relevant to the large production of these fungal products.

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Materials and Methods

Collection of sclerotia. Sclerotia of *G. umbellata* were collected from Shan-xi Sheng, China in April, 1999. The sclerotia collected were inoculated on the surface of wood logs occupied by rhizomorphs of *Armillariella mellea*, buried at the soil depth of about 15 cm and have been cultivated for 2 years under the natural condition in Yangpyung, Kyunggido. To examine morphological characteristics in the sclerotial development, sclerotia of *G. umbellata* were collected from their cultivation area.

Microscopic observation. The developmental process of sclerotia was examined under light and scanning electron microscope. To perform light microscopic observation, the collected samples were washed twice with 0.1 M sodium phosphate buffer (pH 7.2) for elimination of impurities. The washed samples were cut into small pieces of about 1 cm^3, embedded in a freezing tissue embedding medium, and then sectioned in the range of 7 to 10 μm thickness at −20°C with a cryomicrotome (Leica CM 1900; Leica, Germany). Sections were mounted on glass slides and stained using water-iodine solution (25% Gram's iodine in distilled water), Melzer's reagent (1.5% iodine and 5% potassium iodide in distilled water), 0.4% trypan blue solution (Sigma T8154, USA) and lactophenol cotton blue solution (lactic acid/phenol/glycerol/distilled water, 1 : 1 : 2 : 1). The stained specimens were examined with a light microscope (Optiphot-2; Nikon, Japan) and photographed using a camera (Dark box FX-35DX; Nikon, Japan) and a photomicrographic system attachment (Microflex UFX-

DX; Nikon, Japan).

To investigate sclerotial samples of *G. umbellata* with scanning electron microscopy, the collected samples were cut into pieces with 5 mm^3 under the stereo microscope (Leica MZ APO; Leica, Germany). The excised specimens were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), rinsed twice for 30 minutes in 0.1 M sodium phosphate buffer (pH 7.2) and post fixed at 4°C for 2 hours at 1% osmium tetroxide (OsO_4) in 0.1 M sodium phosphate buffer (pH 7.2). Post fixed specimens were rinsed 3 times for 30 minutes with 0.1 M sodium phosphate buffer (pH 7.2), and then dehydrated 3 times with every 10 minutes in a graded ethanol series such as 50%, 70%, 80%, 90%, 100% and 100%. The ethanol was substituted by changes of ethanol and isoamyl acetate at 1 : 3, 1 : 1, 3 : 1 and two changes of 100% isoamyl acetate for every 20 minutes. The specimens were dried using a critical point dryer (Hitachi HCP-2; Hitachi, Japan) with liquid CO_2 as the transition fluid. The dehydrated specimens were mounted on aluminum stubs and coated with gold using a sputter coater (Hitachi E-1010; Hitachi, Japan). The specimens were examined and photographed using a scanning electron microscope (Hitachi S-2380N; Hitachi, Japan) operating at 10 and 20 KV (Zarani and Christias, 1997).

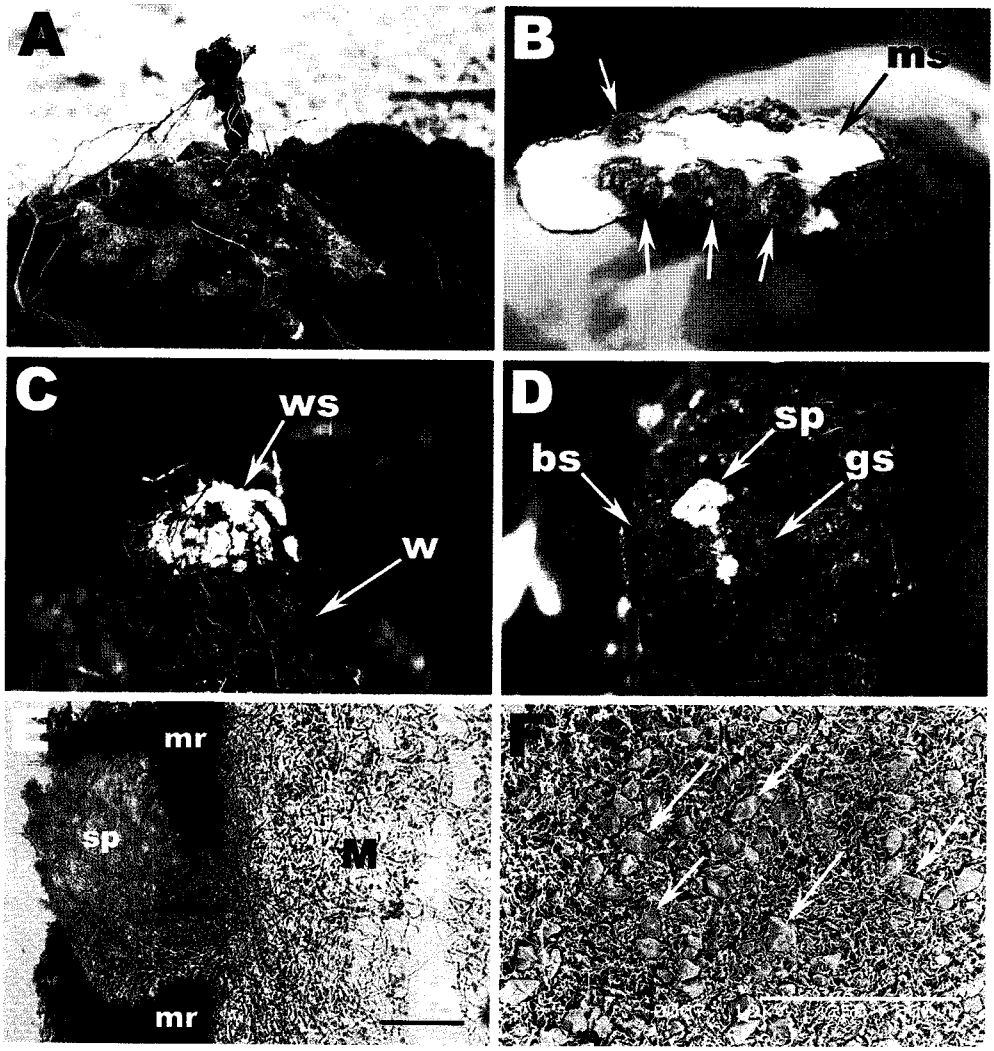

Fig. 1. Morphological features and characteristics of sclerotial development of *Grifola umbellata*. **A**, Morphology of matured sclerotium one year after cultivation in nature. **B**, Cross section of mature sclerotium (ms). Arrows indicate black surroundings formed by invasion of rhizomorphs of *Armillariella mellea* into sclerotium of *G. umbellata*. **C**, Development of white sclerotium (ws) on the wood log (w) colonized by rhizomorphs of *A. mellea* (r) in cultivation area. **D**, Developmental process and maturation of sclerotium of *G. umbellata*. bs = black sclerotium used for inoculum; gs = gray sclerotium developed newly from inoculum; and sp = sclerotial primordia derived from the mature sclerotium for developing new sclerotium. **E**, Sclerotial primordium (sp) emerging out of the melanized rind (mr) of gray sclerotium for initiation of development of new sclerotium. M = medullary layer. Scale bar = 100 μm. **F**, Crystals (arrows) observed in the medullary layer of gray and black sclerotia.

Results

Morphological characteristics of sclerotium of *G. umbellata*. The morphology of sclerotia of *G. umbellata* collected in nature (Shan-xi sheng, China) was similar to that of *G. umbellata* cultivated artificially in Korea. The sclerotia were round to ellipsoid tuber with approximately 15~30 × 10~20 × 1~5 cm in size and multi-branched. The surface of sclerotia was bumpy, rugged and solid. The sclerotia weighed between 100~250 g (Fig. 1A). When the sclerotium of *G. umbellata* was cross sectioned, the internal part of sclerotium was milky white in color and the texture of tissue was very tough and woody (Fig. 1B). Particularly, the many traces formed by invasion of rhizomorph of *A. mellea* were observed in an interior tissue of sclerotium (Fig. 1B).

Development and maturation of sclerotia of *G. umbellata*. The sclerotial development of *G. umbellata* was divided into three stages: In the initial stage, *G. umbellata* formed a white fungal mass by mycelial aggregation (Fig. 1C). The surface of white mycelial mass was

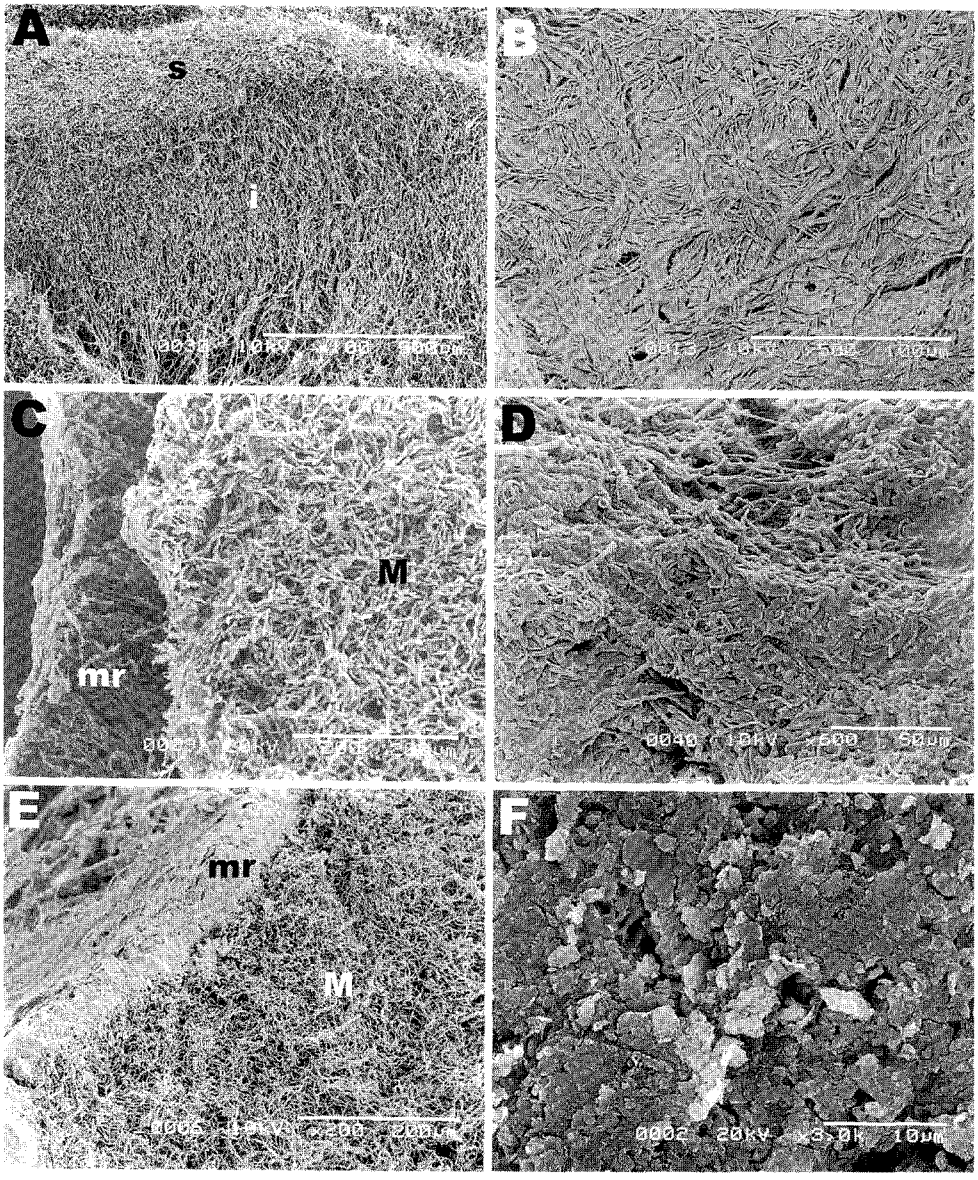

Fig. 2. Sclerotial morphogenesis in *Grifola umbellata* and sequences of structural changes leading to its sclerotial development and maturation. **A**, Cross section of sclerotium in the initial stage. A defined mycelial mass shows a discrimination of interior part (i) and superficial layer (s). **B**, Rind-like structure of the white sclerotium formed by the aggregation of fungal mass in the initial stage of sclerotial formation. **C**, Cross section of gray sclerotium. Melanized rind (mr) and medullary layer (M) are distinguished separately. **D**, Rind structure of gray sclerotium by twined and compact fungal masses in the second stage of sclerotial development. **E**, Cross section of the mature sclerotium. Melanized rind layer (mr) and medullary layer (M) are completely distinguished. **F**, Superficial structure composed of melanized rind layer of the mature sclerotium.

composed of the aerial mycelia for continuous growth (Fig. 2A). Particularly, the white mycelial mass formed a rind-like structure by mycelial aggregation on the parts contacted with impurities such as sand, root and rhizomorph of *A. mellea* (Fig. 2B). In the developmental stage, the white fungal mass was developed into gray sclerotium through differentiating a melanized rind layer on its superficies. The surface of gray sclerotium was soft, and gray to light brown in its color. The structure of gray sclerotium was divided into melanized rind and medulla layer whose mycelial density was more compact than that of the white mycelial mass (Fig. 2C). The structure of rind layer was composed of fungal mass deformed, twined and aggregated each other (Fig. 2D). In the maturation stage, the gray sclerotium was specialized into black sclerotium (Fig. 1A). The surface of mature sclerotium was solid, and composed of dark-colored round or ellipsoid tuber. Tissue structure shown by the cross section was similar to that of gray sclerotium except for rind layer (Fig. 2E). It was observed in the rind layer of black matured sclerotium that the shape of mycelia was entirely changed into different structure like flat crystalline and piled up with many folds (Fig. 2F). Thickness of rind layer was observed in the range of 50~60 μm. Particularly, it is noteworthy that numerous crystals were present in the medullary layer of mature sclerotium (Fig. 1F).

Development of primordium from the mature sclerotium. The mature sclerotium of *G. umbellata* formed a primordium to develop new sclerotium on its superficial part (Fig. 1D). When the structure of primordia was investigated with light microscope, these primordia burst out the melanized rind layer of mature sclerotium with very high mycelial density (Fig. 1E). It was possible to confirm that this fungal structure has been gradually developed into gray sclerotium and then matured into black sclerotium. The mature sclerotium has been grown with repetition of such processes as sclerotial primordium, gray and black sclerotium.

Discussion

The studies of *G. umbellata* have been reported to form underground sclerotia in its life cycle and to derive fruiting body from its sclerotium (Imazeki and Hongo, 1989; Mao and Jiang, 1993). The sclerotial characteristics of *G. umbellata* were similar to those of other fungi except for the formation of a large prismatic crystal structure and thick-wall cells in the middle of hyphae (Guo and Xu, 1991, 1992a). In general higher fungi, the developmental stages in sclerotium formation were distinguished by three stages such as sclerotial initials, developmental and maturation stages (Chet and Henis, 1975; Willettes, 1978; Zarani and Christias, 1997). Guo and Xu (1991) discriminated the sclerotial development of *G. umbellata* followed by the change of their color such as white, gray and black. As a result of microscopical examination, the sclerotial structure and developmental process of *G. umbellata* were similar to those of other fungi presented by Willetts and Bullock (1992). Based on three types of sclerotia of *G. umbellata* such as white, gray and black sclerotium, our study could discriminate the characteristics of sclerotial development of *G. umbellata.* In the initial stage, *G. umbellata* formed a white fungal mass namely, the white sclerotium which had a simple structure formed by mycelial aggregation. In the developmental stage, this white sclerotium grew up and developed into gray sclerotium whose structure was discriminated between the medullary layer and the incompletely differentiated rind layer, and also innumerable crystals began to appear in this stage. When the white sclerotium of *G. umbellata* enlarged its size, the white sclerotium seemed to be exposed to various obstructions under soil. We supposed that gray color was originated from the response against an environmental stress. In the maturation stage, the sclerotium of *G. umbellata* changed its color to black and completely differentiated its rind layer.

It was reported that a new layer of thickened and pigmented cells differentiated into rind layer forming mature sclerotia in the developmental stage of sclerotia of higher fungi (Saito, 1974). Willetts (1978) and Huang *et al.* (1993) proved that melanins could contribute to resistance against other environmental factors including irradiation and biological degradation. In this study, we identified the developmental process of the rind layer that the aerial mycelia of *G. umbellata* were aggregated, twined each other, deformed, changed gradually into lignified tissue and then developed into sturdy structure with thick and solid texture. Particularly, there were no mature sclerotia invaded by the rhizomorph of *A. mellea* in cultivation area. It seemed that the rhizomorph of *A. mellea* was impossible to penetrate into the interior part of the mature sclerotium of *G. umbellata* with the completely developed rind layer. Therefore, it seemed that the rind structure in the mature sclerotia of *G. umbellata* had some important roles to protect themselves against other soil microorganisms, to block impurities such as soil particles, to maintain its moisture contents and to resist other environmental stresses.

In the maturation stage, the sclerotia of *G. umbellata* reached to their final shape and compactness, and their rind layer was rough and scaly. Also, the mature sclerotium attained to maximum dimension which could not grow further. However, these black sclerotia of *G. umbellata* initiated new sclerotial primordia to develop new sclerotium on their surface. We confirmed that because the sclerotial primordia of *G. umbellata* had a high growth rate with compact and dense mycelial mass, the

primordia could burst out of the solid rind layer. Also, the mature sclerotium could develop several primordia on its superficial part. The mature sclerotia of *G. umbellata* had been grown continuously by the repetition of three stages such as white, gray and black sclerotia during its life cycle. Finally, the matured sclerotium of *G. umbellata* appeared to show a multi-branched and tuber-like shape.

In general, the sclerotia of higher fungi produced and contained a large amount of mucilage compounds such as low-molecular weight carbohydrates, monosaccharides, disaccharides, polysaccharides and a sugar alcohol (Tourneau, 1979; Willetts and Bullock, 1992). Often, large amounts of mucilages which were considered as polysaccharides were accumulated in sclerotia so that they were suspended in a mucilaginous matrix (Willetts and Bullock, 1992). Therefore, we could suppose that the crystals of *G. umbellata* were presumed as deposited exudates such as polysaccharides in the identity of crystals formed in the medulla layer of *G. umbellata* and these extracellular matrix seemed to be accumulated on the interhyphal spaces in medullary layer as mucilage compounds.

Particularly, it has been known that the sclerotium of *G. umbellata* had a symbiotic relationship with *A. mellea* (Guo and Xu, 1992b). In this study, we observed that the rhizomorphs of *A. mellea* penetrated into the white fungal mass of *G. umbellata* formed newly and then the invasive rhizomorphs were twined with mycelia of *G. umbellata*. Also, most of the harvested sclerotia of *G. umbellata* were surrounded by rhizomorphs of *A. mellea*. When the mature sclerotia were cut with razor, we could observe some black bands formed by the invasion of rhizomorphs in medullary layer of sclerotia. The rhizomorphs of *A. mellea* lost their original form and only remained their traces in medullary layer. In these results, we could suppose that the rhizomorphs of *A. mellea* contacted with *G. umbellata* and invaded into sclerotial initial of *G. umbellata* without rind layer. Also, it could be supposed that the invasive rhizomorphs were surrounded by mycelia of *G. umbellata* and the sclerotia of *G. umbellata* formed a specific structure to protect themselves from rhizomorphs of *A. mellea* in the later stage of sclerotial development. Finally, the rhizomorphs were lysised by *G. umbellata*. Consequently, the rhizomorphs of *A. mellea* seemed to be used as a nutrition for growth of sclerotium. As a result of this process, the mature sclerotia of *G. umbellata* had some black bands formed by the invasion of rhizomorphs in their medullary layer. We named these structures as black surroundings in the mature sclerotium of *G. umbellata*.

Although it was difficult to study the whole life cycle of *G. umbellata* including its fruit body, we can conclude that these results will contribute to resolving some difficulties obstructive to the mass production of sclerotia of *G. umbellata*.

Acknowledgments

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